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In Vitro Activities of Azithromycin and Doxycycline against 15 Isolates of *Chlamydia pneumoniae*

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Fourteen isolates of *Chlamydia pneumoniae*, 12 from clinically ill patients and 2 from subjectively healthy individuals from an area within a 400-km proximity of Gävle, Sweden, and strain IOL-207, originally from the eye of an Iranian child, were tested for susceptibilities to the antibiotics doxycycline and azithromycin. MICs and minimum chlamydiacidal concentrations were found to correlate well with values reported earlier by other investigators. In addition to MIC and minimum chlamydiacidal concentration testing, testing for the viability of *C. pneumoniae* after exposure to antibiotic concentrations as high as 50 mg/liter was carried out by passaging antibiotic-treated, infected cell cultures four times in the absence of antibiotics. It was found that all *Chlamydia* strains were viable after four passages, regardless of the initial antibiotic concentration in the cell culture.

Chlamydia pneumoniae is a common cause of both upper and lower respiratory tract infection and has recently been found to be a causative agent of chronic pharyngitis (4). Recent findings implicate chronic *C. pneumoniae* infection in the development of cardiovascular disease (12, 15). The majority of infections caused by *C. pneumoniae* are likely either subclinical or asymptomatic and do not necessitate a visit to a medical practitioner. Seropositivity is related to age, and *C. pneumoniae* antibodies are a common finding in all populations (13). It has been suggested that the majority of individuals have more than one infection per lifetime (7).

Persistent infections with *C. pneumoniae* have been reported, and it is generally thought that *C. pneumoniae* infections may require longer periods of antibiotic treatment for the resolution of infection (8, 9, 11). The infectious life form of chlamydiae, the elementary body, is resistant to the action of antibiotics, which target cell metabolism or multiplication for activity. The most probable outcome of most treatments is the inhibition and eradication of antibiotic-susceptible chlamydial forms (i.e., reticulate bodies), leaving a number of elementary bodies in infected tissues and such circulating phagocytic cells as alveolar macrophages. In some patients, it would appear that chlamydiae persist, while in others, eradication takes place. Roblin et al. (14) demonstrated that persistence is not secondary to the development of antibiotic resistance in vitro. The mechanisms by which persistent infections are maintained are not understood.

Tetracycline has been considered the drug of choice for chlamydial infections. Use of the drug for children and pregnant women, who are usually treated with macrolide antibiotics, of which the most well known is erythromycin, is not advised. A new azalide, azithromycin, has been found to possess properties which make it a potentially valuable drug for the treatment of *C. pneumoniae* infections. The tissue penetration and half-life of this drug exceed those of previous macrolides, and the tissue concentrations have been found to be 10 times those of erythromycin (5).

We determined the MICs and minimum chlamydiacidal concentrations (MCCs) of azithromycin and doxycycline for 15 strains of *C. pneumoniae* after one passage. Antibiotic-treated cultures were passaged a further four times to assess the long-term effectiveness of a single dose of antibiotic in vitro with regard to chlamydial survival.

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MATERIALS AND METHODS

Antibiotics. Azithromycin dihydrate (Pfizer Corporation) and doxycycline hydrochloride (Pfizer Corporation) were dissolved according to the instructions of the manufacturer (azithromycin in methanol and then in water, and doxycycline in water). Antibiotic suspensions were prepared fresh on each testing occasion and were tested in final concentrations of 50 to 0.016 mg/liter. Stock solutions at 1,000 mg/liter were diluted 1:10, and 1:5 dilutions of this 100-mg/liter solution were made. All dilutions were done with RPMI 1640 cell culture medium without antibiotics. Final antibiotic concentrations were 50, 10, 2, 0.4, 0.08, and 0.016 mg/liter, since the cell cultures already contained 1.0 ml of cell culture medium.

***C. pneumoniae* strains.** Fifteen strains of *C. pneumoniae*, 14 clinical isolates and strain IOL-207 (kindly donated by S. Darougar, London, United Kingdom), were passaged 15 to 20 times in human lung (HL) cells, with the last six passages being done in antibiotic-free cell culture medium. All strains were stored at −70°C until they were tested. Strains 1, 2, 3, 10, 15, 16, and 24 originated from patients with long-standing upper or lower respiratory tract infections, strains 6, 22, and 23 came from patients with chronic pharyngitis, strain 11 came from a patient with pneumonia, and strains 9 and 12 came from asymptomatic carriers. Strains 1 and 19 were isolated from patients who experienced heart irregularities after a respiratory tract infection.

Cell cultures. HL cells were grown in 24-well tissue culture plates with a culture medium that was composed of basal medium Eagle medium with Earle salts (Hyclone), 10% fetal bovine serum (Sigma Cell Culture), and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (Sigma Cell Culture) supplemented with L-glutamine (Sigma Cell Culture) and that was adjusted to a pH of 7.0. The culture medium used for the determination of antibiotic susceptibilities was composed of RPMI 1640 (Sigma Cell Culture) with 2% fetal bovine serum, HEPES buffer, L-glutamine, glucose, and cycloheximide (Sigma Cell Culture) and was adjusted to a pH of 7.0. Test strains were diluted to a predetermined concentration calculated to give 300 to 500 inclusions per well in RPMI medium. Each chlamydial strain was added to three six-well rows on two plates for susceptibility testing; the last row containing chlamydiae was a positive control for growth with no antibiotics added. The fourth six-well row of HL cells on the plate was not inoculated with chlamydiae and served as a negative control. On a third plate, two additional rows of HL cells were inoculated with the same chlamydial suspensions, and medium containing the same antibiotic dilutions was added. This plate was used for the passaging of strains in antibiotic-free media after an initial 3-day incubation with antibiotics so that the MCC after one antibiotic-free passage and the survival rate of *C. pneumoniae* after four passages could be determined. The plates containing HL monolayers inoculated with *C. pneumoniae* were centrifuged at 1,800 × *g* for 1 h at 30°C. The supernatants were aspirated, and RPMI medium with serial dilutions of azithromycin and doxycycline were added to the wells. The plates were incubated in candle jar boxes for 3 days, then stained with a monoclonal antibody specific for *Chlamydia* spp., Pathfinder (Chlamydia Culture Confirmation System; Kallestad), and read with a Zeiss UV microscope. The numbers of inclusions in the duplicate wells containing antibiotic were counted and compared with those in the antibiotic-free monolayers to obtain the MIC for each antibiotic. Each antibiotic-containing culture well was harvested, and the cells were disrupted by vortex mixing, pas-

TABLE 1. MICs and MCCs of azithromycin and doxycycline for 15 isolates of *C. pneumoniae*[a]

Strain	MIC (mg/liter)		MCC (mg/liter)	
	Doxycycline	Azithromycin	Doxycycline	Azithromycin
1	0.08	0.4	0.08	0.08
2	0.06	0.25	0.08	0.4
3	0.4	0.08	0.08	0.08
6	0.08	0.4	0.08	0.08
9	0.08	0.08	0.016	0.08
10	0.08	0.08	0.08	0.4
11	0.02	0.08	0.016	0.08
12	0.25	0.06	0.08	0.08
15	0.08	0.08	0.016	0.4
16	0.01	0.01	0.02	0.04
19	0.02	0.02	0.08	0.04
22	0.08	0.08	0.08	0.4
23	0.08	0.08	0.08	0.4
24	0.08	0.08	0.08	0.4
IOL-207	0.08	0.08	0.02	0.4

[a] The MICs of doxycycline and azithromycin for 90% of the strains were 0.09 and 0.10 mg/liter, respectively; the MCCs of doxycycline and azithromycin for 90% of the strains were 0.06 and 0.21 mg/liter, respectively.

saged once more, and then stained. After that, inclusions were enumerated to arrive at the MCC. A separate set of antibiotic-treated, inoculated monolayers was passaged four times before being stained so that the survival rate of *C. pneumoniae* after exposure to antibiotics could be assessed.

RESULTS

MICs and MCCs of azithromycin and doxycycline were not found to vary significantly for any of the *C. pneumoniae* isolates studied. Values obtained were similar to those reported in other studies (Table 1). Hammarschlag (10) presented a summary of published studies in which MICs varied from 0.031 to 0.5 mg/liter for doxycycline and 0.025 to 1.0 mg/liter for azithromycin and MCCs ranged from 0.05 to 2 mg/liter and 0.125 to 1.0 mg/liter for the respective antibiotics. MICs of doxycycline ranged from 0.008 to 0.4 mg/liter; the MIC for 90% of the strains was 0.09 mg/liter. MICs of azithromycin ranged from 0.01 to 0.4 mg/liter; the MIC for 90% of the strains was 0.104 mg/liter. MCCs ranged from 0.002 to 0.08 mg/liter for doxycycline and 0.08 to 0.4 mg/liter for azithromycin. The MCC of doxycycline for 90% of the strains was 0.06 mg/liter; that of azithromycin for 90% of the strains was 0.21 mg/liter (Table 1). Doxycycline caused an inhibition in the size of inclusions when antibiotic concentrations were close to inhibitory concentrations. This effect was not observed with azithromycin.

All *C. pneumoniae* strains treated with concentrations of antibiotics varying from 50 to 0.016 mg/liter were found to grow well after four passages without antibiotics; the cultures which had been incubated with high antibiotic concentrations grew as well as those incubated with smaller amounts of antibiotics. The ability of chlamydiae to regrow after four passages was not related to the presence or absence of morphologically changed inclusions and was not related to the type of antibiotic used.

DISCUSSION

Doxycycline and azithromycin were found to prevent the multiplication of *C. pneumoniae* at concentrations much lower than those usually found in serum and/or tissues during antibiotic treatment. MICs and MCCs have not been found to be very different irrespective of the methodology used (10). The active multiplication of *Chlamydia* species can easily be demonstrated to be inhibited by antibiotics. It has been reported earlier that tetracyclines cause a decrease in the size of the chlamydial inclusion, and some speculation has been made as to the viability of these small forms. We believe these forms to be viable, since all cultures regrew after four passages in antibiotic-free media after an initial exposure to various concentrations of antibiotics (up to 50 mg/liter).

The chlamydial elementary body, the infectious yet inert form of the bacterium, is resistant to the action of antibiotics that interfere with cell metabolic activities. These particles have been shown to be capable of existing and even multiplying in macrophages and endothelial cells. They may be capable of persisting in the nonreplicating form in vivo for long periods of time. We believe that the most effective way to treat *C. pneumoniae* infections, which have been shown to be difficult to treat in some patient categories, may be by instituting intermittent periods of antibiotic therapy aimed at eliminating actively growing chlamydial organisms throughout several growth cycles.

A direct comparison of activities cannot be extrapolated from in vitro systems; although doxycycline and azithromycin are both active against intracellular pathogens, they have different kinetics and modes of action in vivo. Azithromycin is not found to any appreciable extent in serum but is instead concentrated in tissue, in which it has been found to attain a concentration 10 times greater than that attained by erythromycin and to have a half-life 7 times longer than that of erythromycin (5). Azithromycin has not been found to exert any negative effects on phagocyte cell functioning (6), and it has been postulated that the phagocytic cell, with its increased concentration of antibiotic, may act to deliver active antibiotic to sites of infection.

Doxycycline is found in serum and penetrates tissues and cells. We have shown earlier that doxycycline may exert a slight inhibitory effect on neutrophil and lymphocyte cell functioning in vitro at levels of the drug that are easily attainable in serum (1, 2). If this is true for the in vivo situation, tetracyclines may have a slight anti-inflammatory effect, which may be advantageous in some clinical situations.

The determination of MICs and MCCs for an organism such as *C. pneumoniae* has very little relevance to the in vivo situation, and as has been reported by Roblin et al. (14), persistence does not seem to depend on the development of resistance to antibiotics. Block et al. (3) reported the finding that clarithromycin was at least 10-fold more active against *C. pneumoniae* than erythromycin in vitro, but no difference in treatment efficacy was seen. The validity of using such terminology as "MCC" should be discussed. We were able to show that even after treatment with high concentrations of antibiotics, all strains survived and regrowth was found to occur. The use of MICs and MCCs for a drug such as azithromycin, for which the actual concentrations available for use at the site of infection are much greater, are not valid for comparisons of different antibiotics. In a few cases, the MCC, measured after one passage, was lower than the MIC. This phenomenon may be a result of temporary damage caused by the antibiotics, resulting in difficulty in growing after exposure to even low antibiotic concentrations, but was not an indicator of a lack of bacterial viability, since all strains grew well after four passages.

An intact immune system is most probably the most important parameter for the outcome of treatment, the removal of organisms by phagocytic cells, and the removal of phagocytic cells from the circulation. Infections caused by *C. pneumoniae* may, in many cases, resolve or become asymptomatic without antibiotic treatment. Despite this, new antibiotic treatment

strategies may have to be defined for complicated or persistent infections. One of these strategies may be the use of intermittent periods of antibiotic treatment to prevent the elementary body forms from multiplying and reestablishing an infection after the termination of a course of antibiotic treatment.

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